Molecular pathogenesis of human hepatocellular carcinoma

Snorri S. Thorgeirsson[1] & Joe W. Grisham[1,2]

Hepatocarcinogenesis is a slow process during which genomic changes progressively alter the hepatocellular phenotype to produce cellular intermediates that evolve into hepatocellular carcinoma. During the long preneoplastic stage, in which the liver is often the site of chronic hepatitis, cirrhosis, or both, hepatocyte cycling is accelerated by upregulation of mitogenic pathways, in part through epigenetic mechanisms. This leads to the production of monoclonal populations of aberrant and dysplastic hepatocytes that have telomere erosion and telomerase re-expression, sometimes microsatellite instability, and occasionally structural aberrations in genes and chromosomes. Development of dysplastic hepatocytes in foci and nodules and emergence of hepatocellular carcinoma are associated with the accumulation of irreversible structural alterations in genes and chromosomes, but the genomic basis of the malignant phenotype is heterogeneous. The malignant hepatocyte phenotype may be produced by the disruption of a number of genes that function in different regulatory pathways, producing several molecular variants of hepatocellular carcinoma. New strategies should enable these variants to be characterized.

Hepatocellular carcinoma (HCC) is one of the most frequent visceral neoplasms worldwide, with an estimated 564,000 new cases and almost as many deaths[1,2] in 2000. Much is known about the development and causes of HCC. It nearly always develops in the setting of chronic hepatitis or cirrhosis, conditions in which many hepatocytes are killed, inflammatory cells invade the liver and connective tissue is deposited.

These changes drastically alter the matrix and microenvironment of the liver[3–5]. The initial hepatocellular alterations that precede the appearance of HCC include foci of phenotypically altered hepatocytes and, subsequently, dysplastic hepatocytes that form foci and nodules[3–5]. Some agents that trigger HCCs, and account for much of the marked variation in its incidence, have been identified and their impacts quantified[4]. The various causes of HCC are perhaps better understood than those of any other major cancer in humans. Furthermore, the main causative agents—hepatitis B virus (HBV), hepatitis C virus (HCV) and aflatoxin B1 (AFB)—which together are responsible for about 80% of all HCCs in humans[4], leave 'molecular marks' on hepatocytes that enable the causes of individual HCCs to be determined accurately in many instances[3,6]. Genomic aberrations have been described in thousands of HCCs from humans, yielding a rich (although still incomplete) database[3,5]. The molecular interactions between hepatocytes and the specific etiologic agents of HCC, and how these interactions disrupt hepatocellular genes and gene products leading to the development of HCC, are being elucidated[3,6–8]. Nevertheless, the molecular pathogenesis of HCC—that is, the specific genomic alterations that drive its development—is not understood. (The term genomic alteration is used here to include both quantitative changes in gene expression occurring in the absence of structural abnormality, and qualitative changes in gene expression resulting from aberrations in gene structure.)

Here we summarize data from published studies and correlate the results with specific categories of preneoplastic lesions and with HCC. We have pooled data from many published studies, cited in a recent comprehensive literature review[3] and in more recent publications, in which the categories of the preneoplastic conditions (for example, chronic hepatitis and cirrhosis) and the categories of hepatocellular lesions (for example, cirrhotic nodules or phenotypically altered, adenomatous, dysplastic and/or malignant hepatocytes) have been described. We have also pooled data from HCCs categorized by histological grade according to the universally applied method of Edmondson and Steiner[3]. We have subjected the pooled data to statistical analysis by type of preneoplastic condition or hepatocellular lesion. We have included all studies that met these minimal criteria, in order to evaluate the heterogeneity of the results of numerous studies from different geographic regions.

Our analysis outlines a general developmental sequence of genomic changes during the process of hepatocarcinogenesis and indicates molecular pathways that may drive hepatocytes to develop a malignant phenotype. Accelerated proliferation of hepatocytes and development of monoclonal hepatocyte populations occur in all preneoplastic conditions and altered cell populations, and these changes continue in HCCs. Genomic alterations appear to develop randomly, beginning in preneoplastic lesions, and their development escalates in dysplastic hepatocytes and HCCs. The extensive heterogeneity of genomic lesions displayed by HCCs suggests that HCC may be produced by selection of both genomic and epigenetic alterations that compromise more than one regulatory pathway. Additional data are needed to fully understand the molecular pathogenesis of HCC, however, and we suggest specific studies and new strategies.

[1]*Laboratory of Experimental Carcinogenesis, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA.* [2]*Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA. Correspondence should be addressed to S.S.T. (e-mail: snorri_thorgeirsson@nih.gov).*

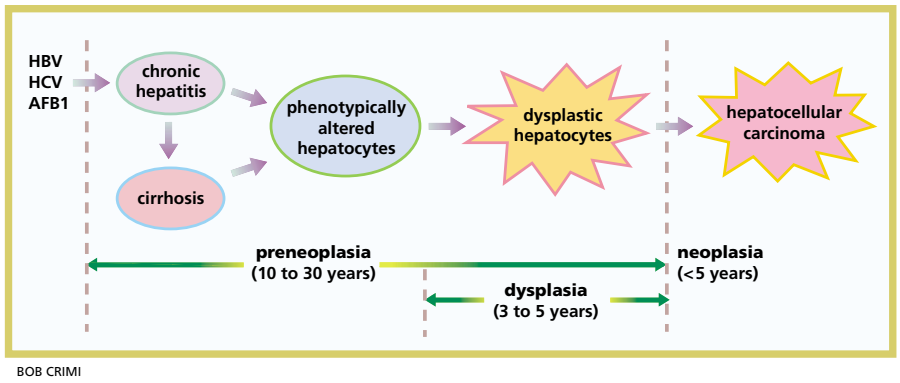

Fig. 1 Chronologic sequence of cellular lesions culminating in the development of hepatocellular carcinoma in human.

Sequential morphological changes in the liver leading to HCC

Hepatocarcinogenesis in humans unfolds during a process that may take more than 30 years after chronic infection with HBV or HCV is first diagnosed[3,9] (Fig. 1). Cirrhosis and HCC occur routinely in a fraction of patients who develop chronic infection with HBV or HCV. HCCs arise with increasing frequency in livers that are the site of chronic hepatitis[10,11] and cirrhosis[12–16], particularly from dysplastic hepatocytes[3,17,18]. The tissue lesions that commonly precede HCC (chronic hepatitis and cirrhosis containing foci of phenotypically altered and dysplastic hepatocytes) provide a framework for identifying the temporal order with which genomic alterations develop during hepatocarcinogenesis (Fig. 1), the first step in defining the molecular pathogenesis of this neoplasm.

Genomic changes during the preneoplastic plastic phase

During most of the long preneoplastic stage leading to HCC, alterations in gene expression are almost entirely quantitative, occurring by epigenetic mechanisms in the absence of detected changes in the structures of genes or chromosomes. Elevated expression of transforming growth factor-α (TGF-α)[3,19] and insulin-like growth factor-2 (IGF-2)[3] is responsible for accelerated hepatocyte proliferation. Altered expression of TGF-α and IGF-2 reflects the expression of several genes that are strongly upregulated by epigenetic mechanisms during the preneoplastic stage (Fig. 2). Upregulation of TGF-α and IGF-2 results from the combined actions of cytokines produced by chronic inflammatory cells that infiltrate the carcinogen-damaged liver[3], viral transactivation[3,6–8] and the regenerative response of the liver to cell loss[3]. Dysregulated expression of IGF-2 is associated with altered methylation and re-imprinting of the IGF-2 gene[3,20], inactivation of the P1 promoter, activation of the P3 promoter and production of large amounts of this growth factor (ref. 3). Hepatocytes proliferate repeatedly[3,21,22] and monoclonal hepatocyte populations develop[3,23–26] (Fig. 2), along with progressive telomere shortening[3] (data not shown) and re-expression of the telomerase enzyme complex[3,27–29] (Fig. 3).

Aberrant methylation (hypo- or hypermethylation) also alters CpG groups of other genes and chromosomal segments, beginning in livers that are the sites of chronic hepatitis and cirrhosis[3,30–41] (Fig. 3). Expression of DNA methyltransferases (DNMTs), which catalyze the methylation and demethylation of CpG groups, is increased in a fraction of livers affected with chronic hepatitis and cirrhosis[3,40,41]. Both DNMT1 and DNMT3a are strongly upregulated in HCCs[40,41]. *S*-adenosylmethionine synthase and glycine *N*-methyltransferase[3], which augment the hepatocellular pool of methyl groups available for methylation reactions, also are upregulated in many HCCs. Other possible causes of epigenetic changes in gene expression include changes in chromatin acetylation, which has apparently not been examined in livers during HCC development, and cis- and transactivation of genes resulting from the actions of viral transactivating molecules, integrated viral promoters, transposed cellular promoters, or some combination thereof[3,6–8].

Microsatellite instability occurs in hepatocytes in some chronic hepatitis, cirrhosis and HCC[3,36–48], but aberrations in other types of DNA repair in HCCs have not been reported. Structural alterations (such as amplifications, mutations, deletions and transpositions) develop slowly in a few genes and chromosomal loci during the early preneoplastic phase, but increase markedly in dysplastic hepatocytes and HCCs. Simultaneous examination of multiple loci detects allelic deletions in 30–50% of livers with chronic hepatitis or cirrhosis[36,43,44,47], in 70–80% of dysplastic

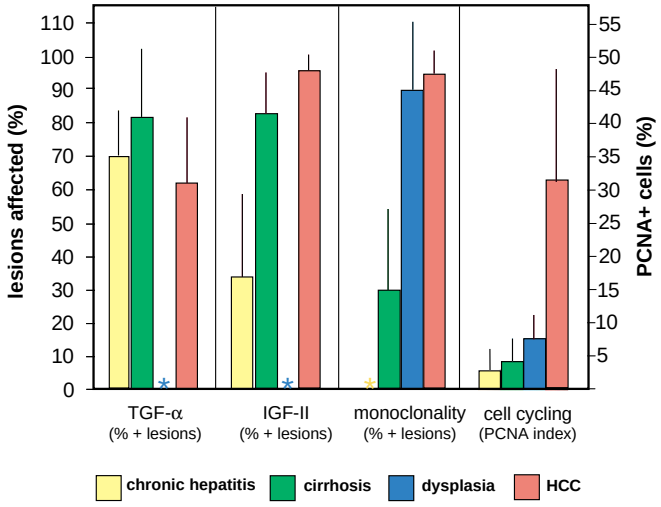

Fig. 2 Changes in expression of TGF-α and IGF-2, monoclonality and cell cycling in hepatocytes from livers that were the site of chronic hepatitis (yellow) and cirrhosis (green), in dysplastic hepatocytes (blue) and in HCC (red). Data in this and other figures and tables are presented as means ± s.d. The data for TGF-α and IGF-2 indicate the fraction of lesions that were histochemically positive (+) for each growth factor. In both instances, the histochemical findings were supported by the relative expression of growth-factor mRNA, quantification of growth-factor protein or both. Asterisks in the bars for TGF-α and IGF-2 for dysplasias indicate insufficient data to quantify, although evidence suggests that both growth factors are highly overexpressed in dysplastic lesions. Monoclonality was determined from the pattern of integration of HBV DNA into hepatocyte DNA, the random silencing of a polymorphic X-linked gene in females, DNA fingerprint patterns obtained by PCR amplification of multilocus minisatellite probes or random primers, or combinations of these factors. Hepatocytes containing proliferating cell nuclear antigen (PCNA) were identified by immunohistochemistry.

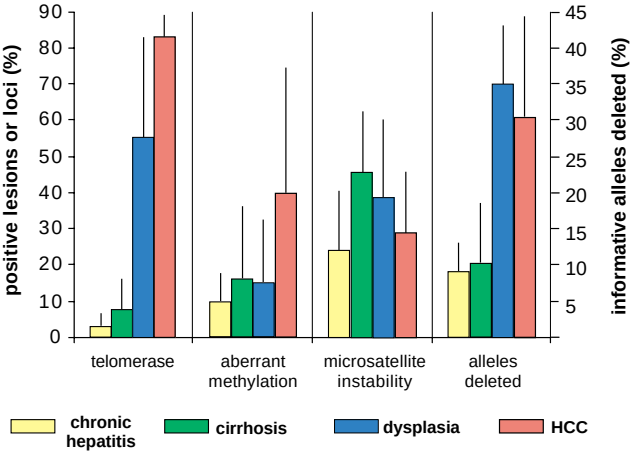

Fig. 3 Changes in telomerase expression, aberrant gene methylation, microsatellite instability and fraction of alleles deleted in hepatocytes from livers the site of chronic hepatitis (yellow) and cirrhosis (green), dysplastic hepatocytes (blue) and HCC (red). Telomerase was detected by conventional TRAP assays (usually with internal standard) or by semiquantitative assays. These assays were supported by RT–PCR of telomerase or hTERT mRNA. Microsatellite instability indicates the fraction of lesions that showed at least one unstable locus. Deleted alleles refers to the fraction of lesions that showed the loss of one allele at one or more loci.

nodules[44,49] and in almost all HCCs[3,5,36,43,44]. Among multiple loci studied simultaneously, however, the proportion of informative alleles that are deleted in chronic hepatitis and cirrhosis is much lower than in either dysplastic hepatocytes or HCCs[3,5,36,43,44,47] (Fig. 3). Thus, the multiplicity of allelic deletions in affected cell populations is low in chronic hepatitis but rises sharply in dysplastic hepatocytes, and is highest in HCCs. Both microsatellite instability at identical loci[3,5] and identical allelic deletions or gene mutations[3,5,36,44,49,50] have been described in cirrhotic and dysplastic nodules and adjacent HCCs, indicating that HCCs often arise as clonal outgrowths of cirrhotic (dysplastic) nodules. Several of the structurally aberrant loci detected in preneoplastic cell populations differ from those found in adjacent HCCs[3,36,47], however, suggesting that many of the cells harboring the early genomic aberrations do not evolve into the malignant phenotype.

Genomic changes in HCC

The early epigenetic changes and some of the early structural alterations of genes or loci are not sufficient to induce malignant phenotypes in hepatocytes, as HCCs ultimately develop in only a minor fraction of the livers with chronic hepatitis or cirrhosis. Epigenetic changes in gene expression that continue in HCCs appear to act indirectly by creating conditions that increase the chance of generating hepatocyte populations containing critical combinations of structurally and functionally aberrant genes. HCCs have recurrent allelic deletions[3,5,36,43,50,51] (Fig. 4) and regional losses and gains[3,5,52–60] (Fig. 5) on several chromosomes. Individual HCCs typically contain multiple allelic deletions and chromosomal losses and gains concurrently; even small, well-differentiated HCCs often contain simultaneous structural alterations in many genes and chromosomes[60].

The potential causes of structural aberrations in genes in HCCs are numerous and varied. In at least some instances epigenetic changes directly precede structural alterations in the same genes. For example, overexpression of the gene c-*myc* in HCCs is initially correlated with promoter hypomethylation and later with gene amplification[3]. Reduced expression of *CDKN2A* (also known as $p16^{INK4A}$) is associated predominantly with promoter hypermethylation[3,5,36,43,50,51], but loss of heterozygosity (LOH), biallelic loss and mutation also are responsible for some loss of expression of this gene[3,5,35,50,61]. Hypermethylation may precede locus deletion, as exemplified by loci located on chromosome 16q[3,34]. Paracentromeric chromosomal translocations, which are frequent in HCCs[62], are also preceded by extensive hypermethylation of these chromosomal regions[36]. Additionally, both HBV and AFB produce structural changes in the genomic DNA[3,6–8], which may induce mutations, breaks and rearrangements. Molecular products of HBV and HCV may impair the function of enzymes involved in DNA repair[3,6–8]. Microsatellite instability reflects impaired DNA mismatch repair and leads to frameshift mutations and deletions[63], and may presage the expression of a 'mutator phenotype' by affected cells[64]. Complete erosion of telomeres, which may exist in highly replicated preneoplastic and neoplastic hepatocytes, results in 'sticky' chromosome ends that are analogous to double-strand breaks and produces mitotic non-disjunction and chromosome disruption[65]. Oxidative and nitrosative damage to the genomic DNA of hepatocytes occurs in the chronically inflamed liver[3,66] and can lead to mutations in genes if not repaired[67]. Oxidative damage also mutates mitochondrial DNA during chronic hepatitis, cirrhosis and HCC[68].

Sequential development of genomic structural aberrations

As indicated by their relative abundance in preneoplastic lesions and HCCs, structural genomic changes (evidenced by allelic deletions and segmental gains and losses) develop slowly during chronic hepatitis and cirrhosis (Fig. 6). Although a few allelic deletions have been detected in chronic hepatitis and cirrhosis[36,43,44,51], chromosome segmental gains and losses have not[3,5,57], perhaps reflecting the relative insensitivity of comparative genomic hybridization (CGH) for detecting small segmental chromosomal lesions. Formation of both allelic deletions[44,49] and segmental gains or losses[3,57] appears to escalate sharply in dysplastic hepatocytes, and multiple alleles or loci are affected simultaneously. Neither the fraction of informative alleles that are deleted nor the number of segmental gains and losses differ

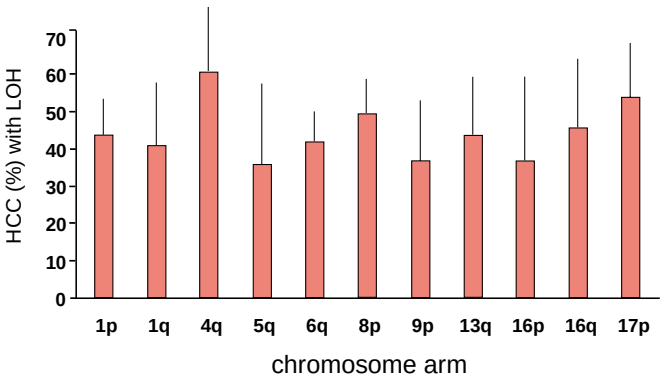

Fig. 4 Autosome arms that contain allelic deletions in more than 30% of reported HCC. LOH was detected by methods employing restriction fragment length or microsatellite polymorphisms. Other autosome arms contain allelic deletions in less than 20% of HCC.

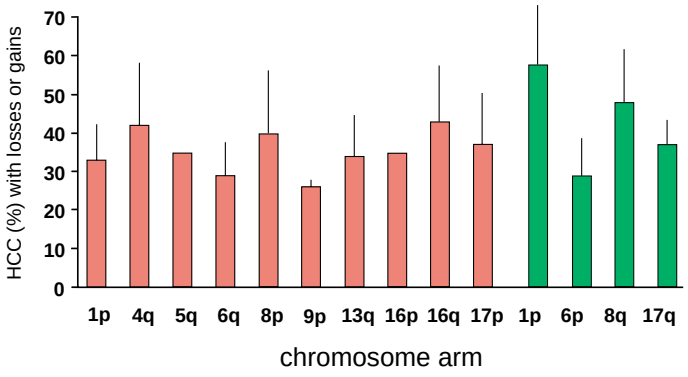

Fig. 5 Autosome arms that show regional losses (red) and gains (green) in more than 20% of reported HCC, as detected by comparative genomic hybridization. In general, regional losses by CGH correspond well with LOH (Fig. 4).

significantly between dysplastic lesions and HCCs (Fig. 6). New structural aberrations develop at some loci as HCCs progress from small, well-differentiated tumors to large, poorly differentiated tumors[3,5,36,51,54,55] (Fig. 7). Production of new structural aberrations in a clone of malignant cells leads to clonal divergence and neoclonal evolution of cells that now contain a more diverse mixture of genomic aberrations.

Heterogeneity of genomic changes in HCC

Although nearly all HCCs contain multiple genes and loci with structural alterations, alterations in individual genes (Table 1)[3,5,34,44,60,68–73], allelic deletions, and losses or gains of specific chromosomal loci rarely affect more than half of HCCs analyzed (Figs 4 and 5). This genomic heterogeneity may result in part from the divergent characteristics of the HCCs that have been studied, which include tumors that are small, well-differentiated and localized as well as those that are large, poorly differentiated and metastatic. But even when the data are segregated according to indicators of HCC progression, including histological grade (Fig. 7) and size or clinical stage (data not shown), there remain high coefficients of variation among results from different studies.

Heterogeneity of genomic aberrations may also reflect the actions of different causative agents. A notable example of an aberration in gene structure related to a specific cause of HCCs is the high frequency of mutations affecting Ser 249 of p53 in the tumors of patients chronically exposed to AFB[3,8,74]. A few studies[54,56] (but not all[3,5,55]) have also suggested that losses and gains of chromosome regions may differ in HCCs caused by HBV as compared to HCV infection. Heterogeneity at the molecular level may also result from the modification of gene function by different mechanisms, including altered methylation, allelic deletion and mutation, among others. Current evidence suggests that the regulatory pathways controlling proliferative cycling, replicative lifespan ('immortality'), death, differentiation and genomic integrity may each be abnormal in some HCCs. Together these regulatory pathways contain thousands of separate molecules whose aberrant expression or structure may subvert precise control of cellular phenotype. The multiple regulatory circuits that control cellular phenotype form complex, redundant networks in which individual pathways interact with one another. Some molecules, such as p53, occupy critical positions at 'nodes' interconnecting several regulatory pathways. An aberration in a single gene whose protein product occupies such a node can impair the control of more than one cellular process and disrupt several regulatory pathways.

The marked variation in expression of specific genetic aberrations in HCCs documented in numerous independent studies, coupled with the limited 'penetration' of any single genomic aberration among all HCCs examined (Table 1; Figs 4 and 5), suggests an alternate possibility to explain molecular heterogeneity. It is possible that a number of different combinations of aberrant genes and regulatory pathways can generate the neoplastic phenotype in hepatocytes, and that this results in several variants or subsets of tumor having different growth properties. This has important implications for accurate molecular diagnosis and prognosis. The design of targeted molecular therapies may need to be tailored to the specific molecular phenotype of the particular HCC. Studies concurrently examining multiple genes in the same HCCs will be required to evaluate this possibility thoroughly. But data already available support the notion that aberrations in different genes and molecular pathways are responsible for the clinical properties of different HCCs. For example, HCCs with mutant β-catenin are clinically less aggressive than those without this mutation but harboring multiple chromosomal aberrations[3,5,72,75]. This difference is probably linked to a particular mechanism of HCC development. Additional studies are needed to identify other molecular variants of HCC and to correlate molecular status with clinical course.

Combinations of genomic aberrations detected in different clonal preneoplastic lesions and in separate HCCs located in the same liver differ from one another. This reflects both the random creation of individual genomic aberrations and the clonal populations that derive from them. Heterogeneity of aberrant genomic combinations in malignant clones supports the idea that diverse combinations of genomic aberrations can sufficiently dysregulate

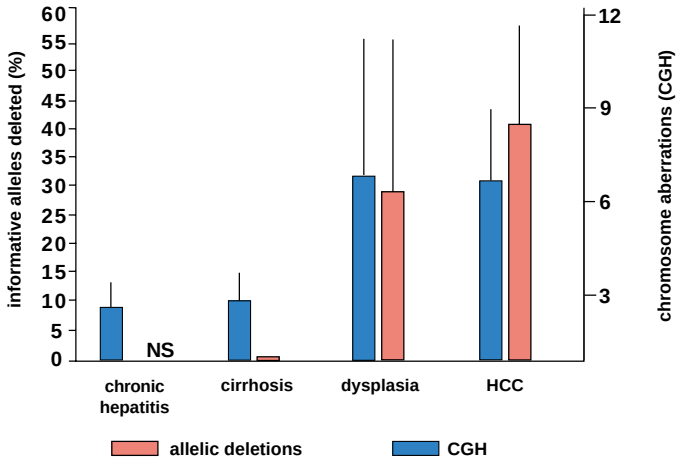

Fig. 6 Sequential development of structural genomic aberrations, as reflected by allelic deletions (red) and chromosome regional losses and gains (green) in hepatocytes in livers the site of chronic hepatitis and cirrhosis, dysplastic hepatocytes and HCC.

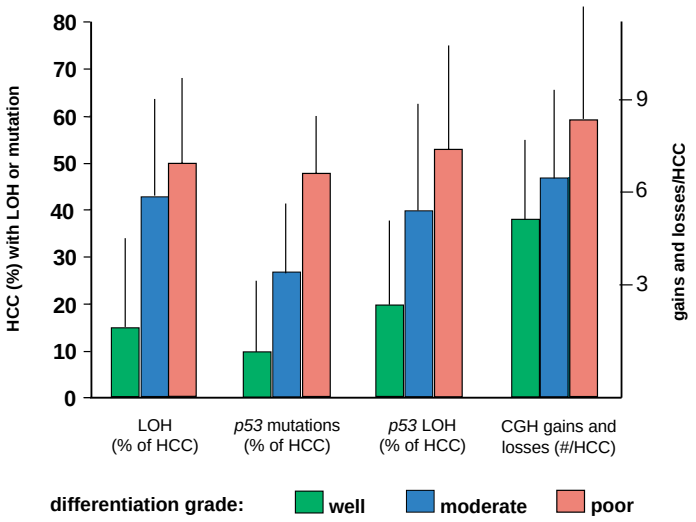

Fig. 7 Accumulation of selected structural genomic aberrations in HCC with progressive deterioration of tumor cell differentiation. Well differentiated (green), moderately differentiated (blue) and poorly differentiated (red) HCC.

hepatocytes that they become malignant. Furthermore, the concurrent presence of genomically distinct malignant clones[3,76] suggests that the entire liver undergoes neoplastic development as a 'field'[77] with multiple hepatocytes progressing to malignancy simultaneously but by different 'genomic routes' and at different rates.

Unidentified genes may be involved in HCC

Recurring allelic losses or gains have been detected on 14 chromosome arms in more than 30% of all HCCs in which allelic deletions[3,5,36,43,49,51] (Fig. 4) and loss or gain of chromosomal regions[3,5,52–60] (Fig. 5) have been analyzed. In theory, the deleted chromosome regions may contain tumor-suppressor genes. Indeed, some frequently deleted chromosome regions contain genes that frequently undergo allelic or chromosome regional loss in HCC (Figs. 4 and 5), such as the tumor-suppressor genes *p53* and *Rb*, which are often inactivated by deletion or mutation (Table 2). The gene *CDKN2A*, which is inactivated by mechanisms including methylation, deletion and mutation, is also located in a frequently deleted chromosomal region[3,5,36,50,61] (Table 1). Likewise, some of the areas of chromosome regional gain (Fig. 5) contain genes encoding such molecules as c-myc, which is overexpressed in most HCCs[3] and is amplified in 30% (refs 3,5; Table 1). New genes that may be involved in the development of HCC have been isolated from a frequently deleted area of chromosome 8p[3,78], but specific HCC-related genes have not been identified in most chromosomal regions that frequently show genomic loss or gain in HCC.

Whether the anonymous genes located in these chromosome regions are causally related to HCC development, or are merely 'bystanders' whose alteration merely reflects widespread chromosome instability in HCCs, is not clear. That the most frequent losses and gains in HCCs involve only a limited number of chromosome arms (Figs. 4 and 5) suggests that each affected region contains one or more genes whose loss or altered function or expression contributes to the development and progression of HCC. It further implies that either the development or persistence of these changes is not random. When the ability of a hepatocyte to control genomic integrity is impaired, genetic structural alterations are likely to occur stochastically in the face of sustained high rates of cell proliferation, coupled with diminished capacity to repair new aberrations. The notion that genetic changes are randomly generated during hepatocarcinogenesis is supported by the high frequency of aberrations in housekeeping genes[3], many of which are not directly involved in the development of a malignant phenotype, and by the progressively increasing number and variety of loci affected as hepatocarcinogenesis continues. Also, of several chromosome arms that contain frequently deleted regions, in a significant fraction of affected HCCs, the entire chromosome arm is lost[3,5]. Frequent loss of entire chromosome arms suggests the action of a process without specificity for particular genes. Hypermethylation of paracentromeric chromosome regions[36] may be the mechanism causing the nonspecific loss of chromosome arms. The most frequent combinations of gene aberrations detected in HCCs may result from the darwinian selection of alleles providing survival and growth advantages from a randomly generated mixture of altered genes. To solve this conundrum and to fully understand the molecular pathogenesis of HCC, it will be necessary to identify the genes contained in the affected chromosome arms and ascertain the specificity of their relationship to the development of HCC.

Focusing future studies

Most previous genomic studies have investigated fully developed HCCs; much less is known about the genetic bases of preneoplastic lesions (foci and nodules of phenotypically altered and dysplastic hepatocytes) and hepatocellular adenomas (HCAs), the benign neoplastic counterpart of HCC. The molecular pathogenesis of HCC cannot be understood without more detailed study of the genomic alterations characterizing its early development and the genomic differences between benign and malignant hepatocellular tumors. The proximate cellular precursors of HCC—foci of phenotypically altered hepatocytes and dysplastic hepatocytes—merit especially intense study. Laser-capture microscopy (LCM) enables these individual lesions to be selectively recovered as cellular clones for genomic analysis[79]. Recent studies of preneoplastic lesions recovered by LCM and similar techniques have yielded provocative results, suggesting that aberrations of particular genes and chromosomes may presage the emergence of HCCs[44,47,49,57], but additional studies are needed.

Table 1 • Genes affected by LOH, mutation, or both (>15% of HCC)

Gene	LOH (%)[a]	Mutations (%)[b]
CDH1	(69)*	—
TP53	42 ± 18	27 ± 18
IGF2R (*M6P*)	42 ± 28	13 ± 20
RB1	35 ± 17	(22)*
CDKN2A (*p16*^{INK4A})	30 ± 22	6 ± 8
PTEN	(30)*	(17)*
DLC1	(44)*	—
TP73	(30)*	—
EXT1	(20)*	—
MLH1	(20)*	—
THRB	—	(76)*
THRA	—	(65)*
E2F4	—	(36)*
CTNNB1	—	17 ± 18

[a]LOH in <15% with regard to *FHIT*, *BRCA1*, *BRCA2*, *NME1*, *MADH4*, *MSH2*, *APC*, *VHL*, *DCC* and *WT1*. No LOH was found with regard to *MCC*, *E2F1* or *p57*. [b]Mutations in <15% in *BRCA2*, *MADH2*, *MADH4*, *TGFBR2*, *AXIN1*, *ARHH*, *SSPN* and *NRAS*. No mutations were found in *APC*. *, mean value calculated from 1 or 2 studies.

HCAs, usually benign, occasionally develop dysplastic changes and transform into HCC[3,80], but neither the molecular basis of HCA nor that of its conversion to HCC is known[3]. Detailed analysis of genomic alterations in HCAs will help clarify the molecular basis of the malignant hepatocyte phenotype. It could also contribute to the understanding of how specific etiologic agents may differentially perturb the molecular regulation of hepatocytes, because the main etiologic agents of HCAs are contraceptive and anabolic steroids[3] rather than the agents responsible for most HCCs.

Most published studies have analyzed only one or two genes or gene products in individual HCCs, and have therefore not demonstrated the potential molecular diversity of this neoplasm. Using gene-expression arrays (cDNA microarrays), it is now feasible to develop gene-expression profiles of neoplastic cells, using RNA isolated from a few cells from each of many HCCs[81]. Similar studies must be extended to the analysis of genomic alterations in preneoplastic lesions. Gene profiling provides a new basis to identify groups of genes involved in specific regulatory pathways that are activated or suppressed in different neoplasms. Several exploratory studies have already used differential display, serial analysis of gene expression and cDNA microarrays to examine differential expression of multiple genes in HCCs[82–90]. Although these studies indicate the promise of these new technologies, the results are tentative and incomplete because of the small number of tissue lesions studied and the limited range of genes examined.

In addition, the use of the adjacent diseased liver tissue as the reference for detecting aberrant gene expression in HCCs confounds the interpretation of most such studies. This prevents the detection of genomic alterations common to both the precursor lesions and the HCCs, making it impossible to identify genes that are first aberrantly expressed in chronic hepatitis and cirrhosis and that may lead to HCC. The practice can also confound the detection of other genomic aberrations, including microsatellite instability and locus deletions, in both preneoplastic lesions and tumors. A pool of non-diseased livers would be a more appropriate reference tissue for detecting functionally and structurally aberrant genes in both preneoplastic lesions and HCCs. Livers harboring chronic hepatitis and cirrhosis or dysplastic lesions could provide reference tissues for detecting stepwise changes during the progression of hepatocarcinogenesis.

Genomic arrays can also be used to assess the copy numbers of specific genes (genomic profiling) simultaneously using DNA collected from many HCCs[91] and to map allelic deletions using single-nucleotide polymorphisms (SNPs)[91,92]. SNPs occur roughly every 1,000 bases throughout the genome and are heterozygous about one-third of the time, and can therefore be used to generate dense allelotypes over the entire genome[91,92]. To date, the most dense microsatellite-based allelotype of HCC published contains only about 250 microsatellite loci, separated by an average distance of 20–50 cM (about 20–50 million bases)[3]. A few chromosome arms have been partly or completely allelotyped with microsatellite sequences located every 2–5 cM[3,5], but identification of the unknown genes affected by allelic deletions will require denser allelotypes. Such identification will be necessary to determine whether the deletions are specific or nonspecific—that is, whether there are particular loci in the minimally deleted regions of affected chromosome arms that are frequently affected, or whether loss of large portions of chromosomes or entire chromosome arms leads to passive (nonspecific) deletion of loci. SNPs can also serve as markers in linkage studies of families and populations, and in association studies in HCC patients and controls to identify genes and gene polymorphisms associated with susceptibility to HCC or with particular etiologic agents[92].

Further studies of genomic alterations in animal models of hepatocarcinogenesis will assist in defining the roles of different aberrant genes and molecular pathways in human HCC development. Animal models of hepatocarcinogenesis have provided reliable guides for identifying the cellular development of HCCs in humans[93–95], and they are essential in modeling the molecular pathogenesis of human HCCs[95,96]. Assessment of individual regulatory pathways is particularly difficult to accomplish using accumulated lesions collected from individual human patients, each of which represents an isolated snapshot of one point in a continuous sequence. The ability to modify specific genes and regulatory pathways in mice by transgenic and knockout strategies is a powerful tool for identifying the genes and pathways involved in the development of HCC. Although molecular genetic studies in animals have not yet provided a precise general model for the molecular pathogenesis of HCC in humans[93–95], this may merely reflect the heterogeneity across species of the molecular genetic changes that can lead to HCC. Animal studies have barely begun to demonstrate the potential heterogeneity of the molecular pathogenesis of HCC.

Although thousands of HCCs have been studied, only fragmentary and incomplete data are available about genomic alterations during the development and progression of HCC in humans. Further studies are needed, especially the analysis of genomic changes in preneoplastic lesions and benign neoplasms and the simultaneous evaluation of multiple genes and regulatory pathways in preneoplastic lesions, HCAs and HCCs. Continuing studies of hepatocarcinogenesis in genetically manipulated experimental animals (using transgenic and knockout strategies) should help to identify critical regulatory pathways involved in hepatocarcinogenesis and to determine their mechanisms of action. But an outline of the molecular pathogenesis of HCC is already discernible in the data now available, and could lead to improved therapy. For instance, strategies to delay or reverse the slowly progressing preneoplastic stage, during which gene function is perturbed mainly by potentially reversible epigenetic mechanisms, may prevent or slow the subsequent development of irreversible structural alterations in HCC-related genes, and thereby prevent the emergence of HCC during a normal lifespan. Many HCCs contain irreversible structural changes in multiple genes involving several regulatory pathways simultaneously. Effective molecular genetic therapy of such genomically complex HCCs presents a severe challenge. By contrast, some HCCs, such as those characterized by β-catenin mutations, may have a more limited repertory of genomic alterations and be more amenable to molecular therapeutic intervention.

URLs. An expanded bibliography and index, listing primary publications cited in the text as ref. 3, is available at the National Cancer Institute Laboratory of Experimental Carcinogenesis website, http://neoplasia.nci.nih.gov.

Received 19 June; accepted 27 June 2002.

1. Ferlay, J., Bray, F., Pisani, P. & Parkin, D.M.J. GLOBOCAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Version 1.0., IARC CancerBase No. 5 (Lyon, IARC Press, 2001).
2. Parkin, D.M., Bray, F.I. & Devesa, S.S. Cancer burden in the year 2000. The global picture. *Eur. J. Cancer* **37**, S4–S66 (2001).
3. Grisham, J.W. Molecular genetic alterations in primary hepatocellular neoplasms: hepatocellular adenoma, hepatocellular carcinoma, and hepatoblastoma. In *The Molecular Basis of Human Cancer* (eds Coleman, W.B. & Tsongalis, G.J.) 269–346 (Humana Press, Totowa, New Jersey, 2001).
4. Bosch, F.X., Ribes, J. & Borràs, J. Epidemiology of primary liver cancer. *Semin. Liver Dis.* **19**, 271–285 (1999).
5. Buendia, M.A. Genetics of hepatocellular carcinoma. *Semin. Cancer Biol.* **10**, 185–200 (2000).
6. Bréchot, C. Molecular mechanisms of hepatitis B and C related to liver carcinogenesis. *Hepatogastroenterology* **45** (Suppl 3), 1189–1196 (1998).
7. Diao, J., Garces, R. & Richardson, C.D. X protein of hepatitis B virus modulates cytokine and growth factor related signal transduction pathways during the course of viral infections and hepatocarcinogenesis (Survey). *Cytokine Growth Factor Rev.* **12**, 189–205 (2001).

8. Smela, M.E., Currier, S.S., Bailey, E.A. & Essigmann, J.M. The chemistry and biology of aflatoxin B1: from mutational spectrometry to carcinogenesis. *Carcinogenesis* **22**, 535–545 (2001).
9. Tong, M.J., El-Farra, N.S., Reikes, A.R. & Co, R.L. Clinical outcomes after transfusion-associated hepatitis C. *New Engl. J. Med.* **332**, 1463–1466 (1995).
10. Takano, S., Yokosuka, O., Imazeki, F., Tagawa, M. & Omata, M. Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. *Hepatology* **21**, 650–655 (1995).
11. Chu, C.M. Natural history of chronic hepatitis B infection in adults with emphasis on the occurrence of cirrhosis and hepatocellular carcinoma. *J. Gastroenterol. Hepatol.* **15** (Suppl) E25–E30 (2000).
12. Oka, H. *et al.* Prospective study of early detection of hepatocellular carcinoma in patients with cirrhosis. *Hepatology* **12**, 680–687 (1990).
13. Ikeda, K. *et al.* A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. *Hepatology* **18**, 47–53 (1993).
14. Kato, Y. *et al.* Risk of hepatocellular carcinoma in patients with cirrhosis in Japan. Analysis of infectious hepatitis viruses. *Cancer* **74**, 2234–2238 (1994).
15. del Olmo, J.A. *et al.* Incidence and risk factors for hepatocellular carcinoma in 967 patients with cirrhosis. *J. Cancer Res. Clin. Oncol.* **124**, 560–564 (1998).
16. Chiaramonte, M. *et al.* Rate of incidence of hepatocellular carcinoma in patients with compensated viral cirrhosis. *Cancer* **85**, 2132–2137 (1999).
17. Takayama, T. *et al.* Malignant transformation of adenomatous hyperplasia to hepatocellular carcinoma. *Lancet* **336**, 1150–1153 (1990).
18. Seki S. *et al.* Outcomes of dysplastic nodules in human cirrhotic liver: a clinicopathological study. *Clin. Cancer Res.* **6**, 3469–3473 (2000).
19. Kawakita, N. *et al.* Analysis of proliferating hepatocytes using a monoclonal antibody against proliferating cell nuclear antigen/cyclin in embedded tissues from various liver diseases fixed in formaldehyde. *Am. J. Pathol.* **140**, 513–520 (1992).
20. Schwienbacher, C. *et al.* Gain of imprinting at chromosome 11p15: a pathogenetic mechanism identified in human hepatocarcinoma. *Proc. Natl Acad. Sci. USA* **97**, 5445–5449 (2000).
21. Soini, Y., Virkajärvi, N., Lehto, V.P. & Pääkkö, P. Hepatocellular carcinoma with a high proliferative index and a low degree of apoptosis and necrosis are associated with a shortened survival. *Br. J. Cancer* **73**, 1025–1030 (1996).
22. Theise, N.D. *et al.* Low proliferative activity in macroregenerative nodules: evidence for an alternate hypothesis concerning human hepatocarcinogenesis. *Liver* **16**, 134–139 (1996).
23. Paradis, V., Laurendeau. I., Vidaud, M. & Bedossa, P. Clonal analysis of macronodules in cirrhosis. *Hepatology* **28**, 953–958 (1998).
24. Ochai, T., Urata, Y., Yamano, T., Yamagishi, H. & Ashihara, T. Clonal expansion in evolution of chronic hepatitis to hepatocellular carcinoma as seen at an X-chromosome locus. *Hepatology* **31**, 615–621 (2000).
25. Paradis, V. *et al.* Clonal analysis of micronodules in virus C–induced liver cirrhosis using laser capture microdissection (LCM) and HUMARA assay. *Lab. Invest.* **80**, 1553–1559 (2000).
26. Okuda, T. *et al.* Clonal analysis of hepatocellular carcinoma and dysplastic nodule by methylation pattern of X-chromosome-linked human androgen receptor gene. *Cancer Lett.* **164**, 91–96 (2001).
27. Kitamoto, M. & Ide, T. Telomerase activity in precancerous hepatic nodules. *Cancer* **85**, 245–248 (1999).
28. Takaishi, H. *et al.* Precancerous hepatic nodules had significant levels of telomerase activity determined by sensitive quantitation using hybridization protection assay. *Cancer* **88**, 312–317 (2000).
29. Toshikuni, N. *et al.* Expression of telomerase-associated protein 1 and telomerase reverse transcriptase in hepatocellular carcinoma. *Br. J. Cancer* **82**, 833–837 (2000).
30. Liu, Y.W., Chang, K.J. & Liu, Y.C. DNA hypomethylation of proliferating cell nuclear antigen gene in human hepatocellular carcinoma is not due to cell proliferation. *Cancer Lett.* **70**, 189–196 (1993).
31. Shen, L. *et al.* Correlation between DNA methylation and pathological changes in human hepatocellular carcinoma. *Hepatogastroenterology* **45**, 1753–1759 (1998).
32. Kanai, Y. *et al.* DNA hypermethylation at the D17S5 locus and reduced *HIC-1* mRNA expression are associated with hepatocarcinogenesis. *Hepatology* **29**, 703–709 (1999).
33. Nagai, H. *et al.* A novel sperm-specific hypomethylation sequence is a demethylation hotspot in human hepatocellular carcinoma. *Gene* **237**, 15–20 (1999).
34. Kanai, Y., Ushijima, S., Tsuda, H., Sakamoto, M. & Hirohashi, S. Aberrant DNA methylation precedes loss of heterozygosity on chromosome 16 in chronic hepatitis and liver cirrhosis. *Cancer Lett.* **148**, 73–80 (2000).
35. JinBaek, M. *et al.* p16 is a major inactivation target in hepatocellular carcinoma. *Cancer* **89**, 60–68 (2000).
36. Kondo, Y. *et al.* Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis—a comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. *Hepatology* **32**, 970–979 (2000).
37. Takai, D., Yagi, Y., Habib, N., Sugimura, T. & Ushijima, T. Hypomethylation of LINE1 retrotransposon in human hepatocellular carcinomas, but not in surrounding liver cirrhosis. *Jpn. J. Clin. Oncol.* **30**, 306–309 (2000).
38. Tchou, J.C. *et al. GSTP1* CpG island DNA hypermethylation in hepatocellular carcinoma. *Int. J. Oncol.* **16**, 663–676 (2000).
39. Kaneto, H. *et al.* Detection of hypermethylation of the *p16^{INK4A}* gene promoter in chronic hepatitis and cirrhosis associated with hepatitis B or C virus. *Gut* **48**, 372–377 (2001).
40. Lin, C.H. *et al.* Genome-wide hypomethylation in hepatocellular carcinogenesis. *Cancer Res.* **61**, 4238–4243 (2001).
41. Saito, Y. *et al.* Expression of mRNA for DNA methyltransferases and methyl-CpG-binding proteins and DNA methylation status on CpG islands and pericentromeric satellite regions during human hepatocarcinogenesis. *Hepatology* **33**, 561–568 (2001).
42. Karachristos, A. *et al.* Microsatellite instability and p53 mutations in hepatocellular carcinoma. *Mol. Cell Biol. Res. Commun.* **2**, 155–161 (1999).
43. Kawai, H. *et al.* Quantitative evaluation of genomic instability as a possible predictor for development of hepatocellular carcinoma: comparison of loss of heterozygosity and replication error. *Hepatology* **31**, 1246–1250 (2000).
44. Maggioni, M. *et al.* Molecular changes in hepatocellular dysplastic nodules on microdissected liver biopsies. *Hepatology* **32**, 942–946 (2000).
45. Park, Y.M. *et al.* Microsatellite instability and mutations of *E2F-4* in hepatocellular carcinoma from Korea. *Hepatol. Res.* **17**, 102–111 (2000).
46. Piao, Z., Kim, H., Malkhosyan, S. & Park, C. Frequent chromosomal instability but no microsatellite instability in hepatocellular carcinomas. *Int. J. Oncol.* **17**, 507–512 (2000).
47. Roncalli, M. *et al.* Fractional allelic loss in non-end-stage cirrhosis: correlations with hepatocellular carcinoma development during follow-up. *Hepatology* **31**, 846–850 (2000).
48. Saeki, A. *et al.* Lack of frameshift mutations at coding mononucleotide repeats in hepatocellular carcinoma in Japanese patients. *Cancer* **88**, 1025–1029 (2000).
49. Sun, M. *et al.* An early lesion in hepatic carcinogenesis: loss of heterozygosity in human cirrhotic livers and dysplastic nodules at the 1p36-p34 region. *Hepatology* **33**, 1415–1424 (2001).
50. Chen, T.C. *et al. p16^{INK4}* gene mutation and allelic loss of chromosome 9p21-22 in Taiwanese hepatocellular carcinoma. *Anticancer Res.* **20**, 1621–1626 (2000).
51. Okabe, H. *et al.* Comprehensive allelotype study of hepatocellular carcinoma: potential differences in pathways to hepatocellular carcinoma between hepatitis B virus-positive and -negative tumors. *Hepatology* **31**, 1073–1079 (2000).
52. Chen, Y.J. *et al.* Chromosomal changes and clonality relationship between primary and recurrent hepatocellular carcinoma. *Gastroenterology* **119**, 431–440 (2000).
53. Guan, X.Y. *et al.* Recurrent chromosome alterations in hepatocellular carcinoma detected by comparative genomic hybridization. *Genes Chromosomes Cancer* **29**, 110–116 (2000).
54. Marchio, A. *et al.* Distinct chromosomal abnormality pattern in primary liver cancer of non-B, non-C patients. *Oncogene* **19**, 3733–3738 (2000).
55. Tornillo, L. *et al.* Marked genetic similarities between hepatitis B virus-positive and hepatitis C virus-positive hepatocellular carcinomas. *J. Pathol.* **192**, 307–312 (2000).
56. Wong, N. *et al.* Genomic aberrations in human hepatocellular carcinomas of differing etiologies. *Clin. Cancer Res.* **6**, 4000–4009 (2000).
57. Zondervan, P.E. *et al.* Molecular cytogenetic evaluation of virus-associated and non-viral hepatocellular carcinoma: analysis of 26 carcinomas and 12 concurrent dysplasias. *J. Pathol.* **192**, 207–215 (2000).
58. Balsara, B.R. *et al.* Human hepatocellular carcinoma is characterized by a highly consistent pattern of genomic imbalances, including frequent loss of 16q23.1-24.1. *Genes Chromosomes Cancer* **30**, 245–253 (2001).
59. Rao, U.N. *et al.* Comparative genomic hybridization of hepatocellular carcinoma: correlation with fluorescence *in situ* hybridization in paraffin-embedded tissue. *Mol. Diagn.* **6**, 27–37 (2001).
60. Wilkens, L. *et al.* Differentiation of liver cell adenomas from well-differentiated hepatocellular carcinomas by comparative genomic hybridization. *J. Pathol.* **193**, 476–482 (2001).
61. Laes, J. *et al.* Alterations in P19ARF in rodent hepatoma cell lines but not in human primary liver cancer. *Cancer Genet. Cytogenet.* **117**, 118–124 (2000).
62. Wong, N. *et al.* A comprehensive karyotype study of human hepatocellular carcinoma by spectral karyotyping. *Hepatology* **32**, 1060–1068 (2000).
63. Strauss, B.S. Frameshift mutation, microsatellites, and mismatch repair. *Mutat. Res.* **437**, 195–203 (1999).
64. Loeb, L.A. A mutator phenotype in cancer. *Cancer Res.* **61**, 3230–3239 (2001).
65. Stewart, S.A. & Weinberg, R.A. Telomerase and human tumorigenesis. *Semin. Cancer Biol.* **10**, 399–406 (2000).
66. Kane, J.M. III *et al.* Chronic hepatitis C virus infection in humans: induction of hepatic nitric oxide synthase and proposed mechanisms for carcinogenesis. *J. Surg. Res.* **69**, 321–324 (1997).
67. Hussain, S.P. *et al.* Increased p53 mutation load in nontumorous human liver of Wilson disease and hemochromatosis: oxyradical overload diseases. *Proc. Natl Acad. Sci. USA* **97**, 12770–12775 (2000).
68. Nishikawa, M. *et al.* Somatic mutation of mitochondrial DNA in cancerous and noncancerous liver tissue in individuals with hepatocellular carcinoma. *Cancer Res.* **61**, 1843–1845 (2001).
69. Yamamoto, H. *et al.* Infrequent widespread microsatellite instability in hepatocellular carcinoma and non-HCC tissues. *Oncology Rep.* **7**, 725–729 (2000).
70. Shi, C.Y. *et al.* Codon 249 mutation of the p53 gene is a rare event in hepatocellular carcinomas from ethnic Chinese in Singapore. *Br. J. Cancer* **72**, 146–149 (1995).
71. Terris, B. *et al.* Close correlation between β-catenin gene alterations and nuclear accumulation of the protein in human hepatocellular carcinomas. *Oncogene* **18**, 6583–6588 (1999).
72. Hsu, H.C. *et al.* β-Catenin mutations are associated with a subset of low-stage hepatocellular carcinoma negative for hepatitis B virus and with favorable prognosis. *Am. J. Pathol.* **157**, 763–770 (2000).
73. Satoh, S. *et al.* AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. *Nat. Genet.* **24**, 245–250 (2000).
74. Stern, M.C. *et al.* Hepatitis B, aflatoxin B1, and *p53* codon 249 mutation in hepatocellular carcinomas from Guangxi, People's Republic of China, and a meta-analysis of existing studies. *Cancer Epidemiol. Biomarkers Prev.* **10**, 617–625 (2001).
75. Mao, T.L., Chu, J.S., Jeng, Y.M., Lai, P.L. & Hsu, H.C. Expression of mutant nuclear β-catenin correlates with non-invasive hepatocellular carcinoma, absence of portal vein spread, and good prognosis. *J. Pathol.* **193**, 95–101 (2001).
76. Wilkens, L., Bredt, M., Flemming, P., Klempnauer, J. & Heinrich Kreipe, H. Differentiation of multicentric origin from intra-organ metastatic spread of hepatocellular carcinomas by comparative genomic hybridization. *J. Pathol.* **192**, 43–51 (2000).
77. van Oijen, M.G. & Slootweg, P.J. Oral field cancerization: carcinogen-induced independent events or micrometastastic deposits? *Cancer Epidemiol. Biomarkers Prev.* **9**, 249–256 (2000).
78. Ng, I.O., Liang, Z.D., Cao, L. & Lee, T.K. *DLC-1* is deleted in primary hepatocellular carcinoma and exerts inhibitory effects on the proliferation of hepatoma cell lines with deleted *DLC-1*. *Cancer Res.* **60**, 6581–6584 (2000).
79. Emmert-Buck, M.R. *et al.* Laser capture microdissection. *Science* **274**, 998–1001 (1996).
80. Tao, L.C. Oral contraceptive–associated liver cell adenoma and hepatocellular carcinoma. Cytomorphology and mechanism of malignant transformation.

Cancer **68**, 341–347 (1991).
81. Liotta, L. & Petricoin, E. Molecular profiling of human cancer. *Nature Rev. Genet.* **1**, 48–56 (2000).
82. Hsu, H.C., Cheng, W. & Lai, P.L. Cloning and expression of a developmentally regulated transcript MXR7 in hepatocellular carcinoma: biological significance and temporospatial distribution. *Cancer Res.* **57**, 5179–5184 (1997).
83. Lau, W.Y. *et al*. Differential gene expression of hepatocellular carcinoma using cDNA microarray analysis. *Oncol. Res.***12**, 59–69 (2000).
84. Honda, M., Kaneko, S., Kawai, H., Shirota, Y. & Kobayashi, K. Differential gene expression between chronic hepatitis B and C hepatic lesion. *Gastroenterology* **120**, 955–966 (2001).
85. Lu, T. *et al*. Application of cDNA microarray to the study of arsenic-induced liver diseases in the population of Guizhou, China. *Toxicol. Sci*. **59**, 185–192 (2001).
86. Okabe, H. *et al*. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. *Cancer Res.* **61**, 2129–2137 (2001).
87. Shirota, Y., Kaneko, S., Honda, M., Kawai, H.F. & Kobayashi, K. Identification of differentially expressed genes in hepatocellular carcinoma with cDNA microarrays. *Hepatology* **33**, 832–840 (2001).
88. Xu, L. *et al*. Expression profiling suggested a regulatory role of liver-enriched transcription factors in human hepatocellular carcinoma. *Cancer Res.* **61**, 3176–3181 (2001).
89. Yamashita, T. *et al*. Serial analysis of gene expression in chronic hepatitis C and hepatocellular carcinoma. *Biochem. Biophys. Res. Commun*. **282**, 647–654 (2001).
90. Pollack, J.R. *et al.* Genome-wide analysis of DNA copy-number changes using cDNA microarrays. *Nat. Genet.* **23**, 41–46 (1999).
91. Lindblad-Toh, K. *et al.* Loss of heterozygosity analysis of small-cell lung carcinomas using single-nucleotide polymorphism arrays. *Nat. Biotechnol.* **18**, 1001–1005 (2000).
92. Risch, N.J. Searching for genetic determinants in the new millennium. *Nature* **405**, 847–856 (2000).
93. Grisham, J.W. Interspecies comparison of liver carcinogenesis: implications for cancer risk assessment. *Carcinogenesis* **18**, 59–81 (1997).
94. Kemp, C.J. Comparative hepatocellular cancer genetics. *Am. J. Pathol.* **154**, 975–977 (1999).
95. Fausto, N. Mouse liver tumorigenesis: models, mechanisms, and relevance to human disease. *Semin. Liver Dis.* **19**, 243–252 (1999).
96. Thorgeirsson, S.S., Factor, V.M. & Snyderwine, E.G. Transgenic mouse models in carcinogenesis research and testing. *Toxicol. Lett.* **112–113**, 553–555 (2000).